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***In vitro* characterization of two novel biodegradable vectors for the delivery of radiolabeled antisense oligonucleotides**

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Abstract

The development of antisense oligonucleotides suitable for tumor targeting applications is hindered by low stability and bioavailability of oligonucleotides *in vivo* and by the absence of efficient and safe vectors for oligonucleotide delivery. Whereas stabilization *in vivo* has been achieved through chemical modification of oligonucleotides by various means, effective approaches to enhance their intracellular delivery are still lacking. In this study we report on the characterization *in vitro* of a fully phosphorothioated 20-mer oligonucleotide, complementary to p21 mRNA, radiolabeled with fluorine-18 using a thiol reactive prosthetic group. The potential of two novel synthetic block copolymers containing grafted polyamines on their hydrophobic blocks for vector assisted cell delivery was studied *in vitro*. Extensive cellular uptake studies were performed in human colon carcinoma cell lines with enhanced or deficient p21 expression to evaluate and compare the uptake mechanism of naked and vectorized radiolabeled formulations. Uptake studies with the two novel biodegradable vectors showed a moderate increase in cell uptake of the radiofluorinated antisense oligonucleotide. The two vectors show, however, promising advantages over conventional lipidic vectors regarding their biocompatibility and subcellular distribution.

Keywords

cancer; gene therapy; molecular imaging; radionuclide; vector

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Disclosure Statement

No financial conflicts of interest exist.

Introduction

Antisense oligonucleotides (AsODNs) are being intensively studied for possible applications in cancer therapy and as diagnostic tools to be used for the personalization of treatment approaches in individual cancer patients. AsODNs developed for antisense targeting are generally 18-20 bases in length and undergo Watson-Crick hybridization to targeted mRNA. The specificity for the target mRNA and ease of preparation make AsODNs ideal agents for molecular targeting purposes even though major challenges still hinder their effective use *in vivo*.

Our group is interested in developing a probe for antisense targeting of the cyclin-dependent kinase inhibitor p21, which modulates apoptosis, regulates transcription of genes important in cell cycle progression and senescence, affects DNA repair processes, and potentially also functions as an oncogene. The status of p21 expression shows a potential clinical relevance in chemotherapy of various tumors, including hematologic malignancies², prostate cancer³, breast cancer⁴ and non-small cell lung cancer⁵. This provides the rationale for targeted gene therapies which focus on p21. Downregulation of p21 also shows potential in improving radiocurability⁶. A non invasive method to monitor the p21 status *in vivo* could be predictive of the outcome of cancer therapy. Successful antisense imaging of p21 at the mRNA level has already been reported using an ¹¹¹In-labeled anti-p21 ODN in animals bearing human breast cancer cells that were induced to overexpress p21 by intratumoral injection of epidermal growth factor⁷. Our aim was to develop a fluorine-18 labeled AsODN probe against p21 mRNA for positron emission tomography (PET) to permit a more sensitive and quantitative detection⁸.

Natural phosphodiester ODNs are known to exhibit low bioavailability⁹ and low stability *in vivo*¹⁰. Due to the negative charge, these polyanions can not cross the cell membrane effectively *in vitro*¹¹. Moreover, ODN can bind to undesired targets, including proteins, such as cell membrane proteins or human serum albumin, and non-target mRNA. To avoid degradation due to nuclease activity, AsODNs with a phosphorothioate backbone (PS-AsODNs) have been developed, and have already shown suitability for therapeutic applications¹². Still, effective delivery systems are required to overcome physical barriers and to enhance the intracellular delivery while decreasing non-specific interactions. Synthetic vectors, such as cationic lipids and polyamines are frequently used for this purpose.

In this study we describe a 20-mer PS-AsODN complementary to p21 mRNA with a phosphorothioate monoester at the 5' end conjugated to the radiofluorinated thiol reactive prosthetic group 2-bromo-*N*-[3-(2-[¹⁸F]-fluoropyridin-3-yloxy)propyl]acetamide ([¹⁸F]FPyBrA). A major aim of this study was to evaluate the vector assisted delivery of this radiolabeled probe using different vectors. Two novel biodegradable synthetic vectors, consisting of micelle assembling block copolymers, were compared with a commercially available liposome formulation (Lipofectin). The novel block copolymers are based on poly(ethylene oxide)-*block*-poly(ϵ -caprolactone) (PEO-*b*-PCL) and are grafted with polyamines on their hydrophobic blocks. Cellular uptake studies with naked and vectorized ¹⁸F-labeled AsODN on human colon carcinoma cell lines with induced or deficient p21

expression¹³ were performed to evaluate the cell uptake mechanism of the naked and vectorized radiolabeled formulations. These studies also evaluated the effect of excess levels of AsODN or random ODN (RdODN), incubation at 4°C and co-incubation with poly-(L-lysine) or heparin sulfate, as well as different cell membrane treatments. Although a lower cellular uptake of the ¹⁸F-labeled PS-AsODN was observed with the new biodegradable vectors in comparison with Lipofectin complexes, the new vectors present a promising novel approach with the specific advantage of a cytosolic delivery of AsODNs, a major requirement for successful antisense targeting in cancer therapy.

Materials and Methods

Chemicals with high analytic purity and materials for cell culture with certified analysis were obtained from various commercial suppliers. A 20-mer DNA with a fully phosphorothioated backbone and a phosphorothioate monoester at the 5' end complementary to p21 mRNA with the sequence 5'-TGT-CAT-GCT-GGT-CTG-CCG-CC-3' (PS-AsODN) and the random sequence 5'-CCG-GTG-AAC-GAG-CGA-GCA-CA-3' (PS-RdODN)⁶, with a purity of >95%, were purchased from the University Core DNA Services (Calgary, Canada). [¹⁸F]Fluoride was produced on a TR-19/9 cyclotron (Advanced Cyclotron Systems, Burnaby, Canada) by the ¹⁸O(p,n)¹⁸F nuclear reaction.

The two recently described biodegradable polyamine grafted PEO-*b*-PCL based copolymers, grafted with spermine (SP) and tetraethylenepentamine (TP), with molecular weight of 8300 and 8500 g mol⁻¹, respectively, were prepared as described previously¹⁴ and are presented in Scheme 1. A commercially available liposome formulation based on a 1:1 mixture of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and the fusogenic lipid dioleoyl phosphatidylethanolamine (DOPE) (Lipofectin reagent) was purchased from Invitrogen Corporation, Carlsbad, CA.

Radiofluorination of PS-AsODN

For the preparation of the ¹⁸F-labeled prosthetic group, 2-bromo-*N*-[3-(2-[¹⁸F]-fluoropyridin-3-yloxy)propyl]acetamide ([¹⁸F]FPyBrA), the *N*-Boc-protected nitro-precursor, [3-(2-nitropyridin-3-yloxy)propyl] carbamic acid *tert*-butyl ester was synthesized according to Kuhnast et al.¹⁵ The synthesis was adapted for the fully automated preparation in a commercially available modular synthesis system (Modular Lab, Eckert & Ziegler, Berlin, Germany)¹⁶. The preparation of [¹⁸F]FPyBrA including online normal phase high performance liquid chromatography (HPLC) purification was performed within 65 min, achieving 10-20% decay corrected yield with ¹⁸F starting activities of 3-5 GBq.

Conjugation of [¹⁸F]FPyBrA (100 - 300 MBq) with the PS-AsODN (200 - 500 µg) was carried out in a 250 µL mixture of 0.1 M phosphate buffered saline pH 7 (PBS) and methanol (1/4, v/v) in a sealed V-vial at 120°C for 30 min. The reaction mixture was purified by gel filtration on a NAP-10 column (SephadexTM G-25 DNA grade, GE Healthcare, Buckinghamshire, UK). The radiochemical purity of the ¹⁸F-labeled PS-AsODN was analyzed by reversed phase HPLC on a Beckman Coulter chromatography system with a Model 126 analytical dual pump, Model 168 Diode Array variable UV detector and ACE *Mate*TM Single Channel Analyzer (Ortec, TN) radiometric detection, equipped with a

Phenomenex Luna C18 column (Semi/Prep; 250 x 10.0 mm, porosity 10 μm) using a gradient system of 0.1 M triethylammonium acetate (TEAA) pH 7 and acetonitrile (ACN) with a flow rate of 3 mL/min and increasing levels of ACN: 0-20 min 10-50%, 20-21 min 50-70%, 21-25 min 70%, 25-26 min 70-10%, 26-30 min 10%. The PS-AsODN concentration was determined from the UV-trace at 254 nm using standard curves and confirmed by UV spectroscopy at 260 nm using a Beckman DU 7400 spectrophotometer.

Preparation and characterization of vectorized radiolabeled PS-AsODN

Assessment of AsODN binding capacity and stability: The non-radiolabeled PS-AsODN (2 μg) was incubated with SP and TP (1-64 μg) at different vector to ODN weight ratios (0:1, 0.5:1, 1:1, 2:1, 4:1, 8:1, 16:1, 32:1) in a total volume of 40 μL , at 37°C for 30 min.

Complexation of PS-AsODN was analyzed by agarose gel electrophoresis (2% agarose gel containing 0.05 mg/mL ethidium bromide) at 130 mV for 15 min, after addition of 10 μL of a solution containing 50% glycerol, 1% bromophenol blue and 1% xylene cyanol FF in TBE buffer. The resulting gels were photographed under UV-illumination. The digitized pictures were analyzed with Scion image analysis software to determine the density of ODN bands.

The binding percentage was calculated based on the intensity of free PS-AsODN in the presence of vector with respect to the intensity of free PS-AsODN in the absence of vector. The stability of the different complexes prepared at optimal vector to ODN ratios was determined by challenge with the competing polyanion heparin in increasing heparin to ODN weight ratios (0:1, 0.025:1, 0.05:1, 0.1:1, 0.25:1, 0.5:1, 1:1, 5:1) at 37°C for 1 h by gel electrophoresis as described above. The stability of naked and vectorized PS-AsODN in PBS containing 1% or 25% fetal bovine serum (FBS) (v/v) after 4 h incubation was investigated by comparing the density of the ODN bands with incubation in PBS alone. ODN binding ability, heparin challenge and stability in PBS/FBS was also studied for Lipofectin as the reference complexing agent.

Preparation of radiolabeled vectorized PS-AsODN formulations: For the reference vectorized product, the Lipofectin reagent was diluted with Optimem medium to a concentration of 100 $\mu\text{g}/500 \mu\text{L}$ and pre-incubated at room temperature (RT) for 45 min. Following addition of 500 μL of a 3 μM solution of [18F]PS-AsODN the solution was incubated for another 15 min at RT. For vectorized formulations with SP and TP 500 μL of a 3 μM solution of [18F]PS-AsODN was incubated with either 16 μL of a 5 $\mu\text{g}/\mu\text{L}$ TP solution in water or 32 μL of the corresponding SP solution at 37°C for 30 min.

Evaluation of cellular uptake

Cell uptake studies were performed on two different human colon carcinoma cell lines: a) HCT116 cells, with transcriptional transactivation of p21 through activation of the tumor suppressor TP53 protein and its translocation to the nucleus (induced by ionizing radiation); and b) 80S4 cells, a derivative cell line of HCT116 cells, where both p21 alleles have been deleted through homologous recombination¹³. Cells were cultured in DMEM/F12 nutrient medium supplemented with L-glutamine, 10% FBS (v/v), 50 IU/mL penicillin G, and 50 $\mu\text{g}/\text{mL}$ streptomycin sulfate at 37°C and humidified atmosphere of 5% CO_2 in air. Cells were seeded in 6-well plates at a density of $1-2 \times 10^5$ cells per well. After 24 h incubation

both cell lines were exposed to 5 Gy ^{60}Co gamma radiation in a GammaCell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada), followed by incubation for another 24 h. The expression of p21 mRNA was confirmed by immunofluorescence analysis with a 1:100 dilution of a mouse monoclonal antibody against p21 (p21^{Waf1/Cip1} sc-817; Santa Cruz Biotechnology, Santa Cruz, California) and a 1:250 dilution of Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Molecular Probes, Eugene, OR) using a previously described protocol¹³.

For cell uptake experiments, cells were washed twice with ice-cold DMEM/F12 medium supplemented with 1% FBS (v/v). Cells were then supplied with fresh medium and incubated with naked and vectorized radiolabeled [18F]PS-AsODN formulations, which were diluted to reach a 50 nM ODN concentration in the assay, and incubated at 37°C for 2 and 4 h in triplicates. For naked [18F]PS-AsODN, cellular uptake at a lower concentration (5 nM) and blocking of the cellular uptake by addition of excess PS-AsODN and PS-RdODN (1 μM) was studied. Cell uptake was interrupted by the removal of the supernatant and two rapid rinses with ice-cold PBS. The cells were recovered from the plates by adding 1 N NaOH. All collected fractions were counted in a Wallac 1840 Wizard 3 automatic gamma counter (Perkin Elmer Life Sciences, Boston, MA, USA) and the cell uptake was calculated. The cell associated radioactivity was expressed in relation to the total activity added (% of total activity). For statistical analysis the independent t-test (significance level 0.05) was used. Inhibition of cellular uptake was evaluated by uptake studies at 4°C, a non-permissive temperature for endocytosis. The role of cell surface proteoglycans in the cellular uptake was explored by co-incubation with either 1 μM heparin sulfate, a free glycosaminoglycan known to inhibit the interaction with proteoglycans, or 300 nM poly-(L-lysine), a cationic polymer mediating vector binding to proteoglycans¹⁷. The relative cell associated radioactivity in comparison to the cell uptake at 37°C without co-incubation was calculated.

Two different cell membrane treatments were used to desorb the fraction of naked or vectorized [18F]PS-AsODN bound to membrane proteins. For this purpose after removal of the supernatant and two rinses with PBS, the cells were additionally treated twice with either 0.04 N sodium acetate adjusted to pH 4.5 or PBS containing 10% FBS (v/v) at RT for 5 min before lysing the cells with 1 N NaOH. The relative retained cell uptake was calculated comparing the retained cell associated radioactivity of treated versus untreated cells.

The cytotoxicity of the PS-ODNs and of the different vectors against HCT116 and 80S4 cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HCT116 and 80S4 cells in DMEM/F12 medium supplemented with 10% FBS (v/v) were seeded in 96-well plates at a density of 4,000 cells per well. After 24 h incubation, the medium was aspirated and replaced by 200 μL fresh medium supplemented with 5% FBS (v/v) containing either PS-RdODN, PS-AsODN, Lipofectin, SP or TP. Each component was added separately in triplicate at different concentrations (25, 5, 2.5, 0.5, 0.25, 0.025 $\mu\text{g/mL}$). After 48 h incubation 20 μL MTT in PBS was added to each well and the plate was incubated for 3 h at 37°C. The medium was removed, DMSO (200 μL) was added and the optical absorbance measured at 570 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) to assess cell viability. The

percentage of the cell growth in relation to the control containing cell culture medium without any of the components was calculated.

Results

Radiofluorination of PS-AsODN

Radiolabeling of the PS-AsODN at the phosphorothioate monoester at the 5' end was carried out through alkylation with [^{18}F]FPyBrA achieving a radiolabeling yield of 51.4-74.4% as assessed by radio-HPLC. The ^{18}F -labeled PS-AsODN was separated from unreacted [^{18}F]FPyBrA by NAP purification, achieving a radiochemical purity of >99%. Quantification of the PS-AsODN amount by HPLC and UV spectroscopy resulted in a specific activity of 0.77-4.44 GBq/ μmol (21-120 Ci/mmol) of the final product. The radiochromatogram and UV-trace at 254 nm after purification are shown in Fig. 1.

Preparation and characterization of vectorized [^{18}F]PS-AsODN

The ability of SP and TP for micelle formation was assessed by gel electrophoresis using non-radiolabeled PS-AsODN. Disappearance of free ODN bands in the agarose gel was used as an indicator for complete complexation (Fig. 2). A vector to ODN ratio of 8:1 was sufficient to obtain >95% complexation of PS-AsODN with TP, whereas for SP a ratio of 16:1 was needed. Also for Lipofectin complexes quantitative PS-AsODN complexation was confirmed.

The analysis of the PS-AsODN release from the different non-radiolabeled vectorized formulations by incubation with competing polyanionic heparin revealed complete release of the PS-AsODN at a heparin to polymer ratio of 0.5:1 for SP and TP, whereas for Lipofectin incomplete release was observed even at a heparin to polymer ratio of 5:1. Gel retardation analysis on complexes incubated in PBS containing 1 and 25% FBS for 4 h revealed a high stability of Lipofectin complexes with values >99%. For naked PS-AsODN we found a densitometric intensity of >99% in PBS/1%FBS in comparison with PBS alone, whereas in PBS/25%FBS a lower intensity of 84.7% was found. For complexes with SP and TP a similar stability was found at different PBS/FBS concentrations with values ranging between 84.8 and 94.2%. Results are summarized in Fig. 3.

Evaluation of cellular uptake

The immunofluorescence analysis 24 h after gamma irradiation confirmed induction of p21 expression in HCT116 cells, but not in 80S4 cells. Representative immunofluorescence images captured using a DAPI-FITC fluorescence filter combination show Alexa Fluor 488 detection in correspondence to DAPI-stained nuclei for HCT116 cells (Fig. 4). Initial cell uptake studies with different concentrations of naked [^{18}F]PS-AsODN in HCT116 cells, showed similar uptake at a concentration of 5 nM ($1.50 \pm 0.10\%$; $n=3$) and 50 nM ($1.42 \pm 0.01\%$; $n=3$). Uptake of [^{18}F]PS-AsODN by HCT116 cells in the presence of excess levels of non-radiolabeled PS-RdODN and PS-AsODN, was reduced by 83% and 84%, respectively. A similar uptake was found for 80S4 cells with $1.28 \pm 0.14\%$ ($n=3$) at 50 nM, which was reduced by 84 and 89% in the presence of excess non-radiolabeled PS-RdODN and PS-AsODN, respectively (Fig. 5). The prosthetic group [^{18}F]FPyBrA alone showed a

very low cell uptake of less than 0.5% (data not shown). The uptake of naked [^{18}F]PS-AsODN increased to some extent when the incubation time was raised from 2 to 4 h in both cell lines, but still remained at values below 2%.

Among the different vectorized [^{18}F]PS-AsODN formulations, the highest uptake was found for Lipofectin complexes showing $15.8\pm0.51\%$ and $15.7\pm0.20\%$ uptake in HCT116 and 80S4 cells after 2 h incubation, respectively. The cell uptake increased significantly after 4 h incubation reaching $28.3\pm0.50\%$ and $29.7\pm0.70\%$ in HCT116 and 80S4 cells. A much lower uptake was observed for complexes with SP and TP. Vectorization with SP resulted in $5.29\pm0.32\%$ and $4.26\pm0.10\%$ for HCT116 and 80S4 cells, respectively, after 2h incubation. Under the same condition, vectorization with TP provided $5.70\pm0.22\%$ and $3.94\pm0.19\%$ uptake in the two cell lines, respectively. The cellular uptake 4 h after incubation did not increase significantly in any of the cell lines under study (Fig. 6). For vectorized formulations with SP and TP a statistically significant higher uptake in HCT116 versus 80S4 cells was found at 2 and 4 h after incubation.

Uptake studies performed at 4°C indicated a temperature-dependant uptake of naked [^{18}F]PS-AsODN. At 4°C a relative cell associated radioactivity of only 52.9-53.5% when compared to incubation at 37°C was found (Table 1). Temperature had a minor effect on the uptake of vectorized [^{18}F]PS-AsODN, where a relative cell associated radioactivity of 77.3-90.7% was observed. Heparin sulfate caused a dramatic decrease in uptake for the three vectorized formulations in both cell lines (Table 1). This effect was more pronounced for vectorization with SP and TP in comparison to Lipofectin complexes. A relative cell associated radioactivity of 21.7-24.9% was observed for co-incubation with heparin sulfate for vectorized formulations with SP and TP versus 37.9-44.4% found for Lipofectin. Co-incubation with poly-(L-lysine) resulted in an increased uptake of naked [^{18}F]PS-AsODN and vectorized formulations with SP and TP, increasing the uptake of naked [^{18}F]PS-AsODN from 1.26-1.41% to 15.2-15.7% and of vectorized formulations with SP and TP from 3.94-5.70 to 13.9-14.4%. PLL did not affect the uptake of Lipofectin complexes, which remained at 13.7-13.9%.

Cell membrane treatments with either 0.04 N sodium acetate pH 4.5 or PBS/10% FBS resulted in a reduction of the cell associated radioactivity in all cases (see Table 2). There were slight differences between the cell lines and in general the treatment with PBS/10%FBS was more effective in reducing the cell associated radioactivity than treatment with sodium acetate. The reduction of the cell associated radioactivity was higher for naked [^{18}F]PS-AsODN and vectorized formulations with SP and TP, resulting in a relative retained cell uptake of 66.2-84.9% with sodium acetate and of 31.6-47.4% with PBS/10% FBS. In contrast, for Lipofectin complexes the relative retained cell uptake was always $>80\%$.

No in vitro cytotoxicity was noted for the PS-RdODN, PS-AsODN and the various vectorization agents at similar concentrations to that used in cellular uptake studies.

Discussion

A number of prosthetic groups have been described for the radiolabeling of ODN with ^{18}F 15,18–25. To date, alkylating agents binding to a phosphorothioate moiety have shown the most promising results26,27. These literature precedents guided our choice of the alkylating agent [^{18}F]FPyBrA for the radiolabeling of PS-AsODN complementary to p21 mRNA. We have recently described the fully automated preparation of this prosthetic group in a commercially available modular synthesis unit16. Radiolabeling of the PS-AsODN proceeded with 50–75% yield as indicated by radio-HPLC of the crude product. The radiolabeling mixture was purified by size exclusion chromatography resulting in pure product approaching 5 GBq/ μmol . The slightly longer retention time of [^{18}F]PS-AsODN compared to the unlabeled PS-AsODN precursor, as shown in Fig. 1, suggests that material of much higher effective specific activity could be isolated, but for the present cell based study the specific activity was sufficiently high.

The preparation of vectorized [^{18}F]PS-AsODN formulations under optimized conditions resulted in stable ODN binding of >90% as confirmed by gel electrophoresis. No cytotoxic effects were observed with the polymer amounts used for cellular uptake studies. All formulations were stable in PBS containing 1% FBS up to 4 h and this FBS concentration was used for cell studies. Heparin challenge indicated that polyamine grafted PEO-*b*-PCL micelles were more prone to dissociation in comparison to Lipofectin complexes.

The cell uptake of naked ODN, in particular PS-ODN, is known to take place by adsorption to cell-surface proteins followed by receptor-mediated endocytosis although it is recognized that also other PS-ODN binding proteins are present on the cell surface28. As expected, uptake of [^{18}F]PS-AsODN in the absence of vectorization occurred to a very limited extent (<2%). This limited uptake was efficiently blocked by co-incubation with excess non-radiolabeled PS-RdODN or PS-AsODN and considerably reduced at 4°C (Fig. 5 and Table 1). This is in accordance with a saturable uptake mechanism by receptor mediated endocytosis9 and suggests that on the surface of the cell membrane there are proteins capable of binding ODN. The percentage of uptake in relation to the total activity added was similar when incubating the cells with a 5 and 50 nM dilution of naked [^{18}F]PS-AsODN and we therefore performed all uptake studies with vectorized formulations at 50 nM ODN concentration. Similar results have been reported in other studies29 and saturation seems to occur only at higher concentrations30.

For vectorized [^{18}F]PS-AsODN we found an increase of the uptake up to 20-fold with Lipofectin complexes and up to 3-fold with SP and TP compared to naked PS-AsODN (Fig. 6). Energy depletion at 4°C had little influence on the uptake of vectorized formulations (Table 1) suggesting that other energy-independent uptake mechanisms in addition to electrostatic adsorption followed by endocytosis, such as fusion with or destabilization of the cell membrane are involved31. An increase of the uptake at the later timepoint of 4 hours as compared to 2 hours was observed only for naked [^{18}F]PS-AsODN and Lipofectin complexes, whereas no significant changes in the uptake were observed for vectorization with SP and TP (Fig. 6). This finding could potentially be explained by an early equilibrium

or saturation mechanism at work with these novel vectors or a slowdown of the endocytosis process in the presence of the PEO shell.

Anionic cell surface proteoglycans, especially heparin and heparan sulfate proteoglycans, have been proposed to be involved in the electrophilic attraction of vector cations on the cell membrane³². We therefore investigated co-incubation with poly-(L-lysine) and heparin sulfate known to mediate and inhibit binding to proteoglycans, respectively¹⁷. When incubating naked [¹⁸F]PS-AsODN and vectorized formulations with poly-(L-lysine), we found increased uptake not only for naked PS-AsODN, but also for vectorized formulations with SP and TP. This effect can be explained by the lower complex stability observed with these vectors allowing competitive interaction with a high molecular weight polymer, i.e. poly-(L-lysine), leading to the formation of more stable complexes with higher net positive charge and enhanced binding to proteoglycans³³. Highly anionic polymers such as heparin sulfate can destabilize the interaction between vector and ODN, resulting in inhibition of transfection. The finding that incubation with heparin reduced the uptake of vectorized formulations with SP and TP to a higher extent than Lipofectin complexes (Table 1) besides inhibition of the interaction with proteoglycans also confirms a higher destabilization of these vectorized formulations. Cell membrane treatments with PBS/10% FBS more efficiently decreased the cell associated radioactivity, and this reduction was more pronounced for vectorized formulations with SP and TP as compared to Lipofectin complexes. There are a number of interpretations for the loss of the cell associated radioactivity including a possible dissociation of extracellular vectorized complexes or a loose attachment to cell surface proteins. The latter explanation seems to be more relevant since a reduction of the cell associated radioactivity was also observed for naked [¹⁸F]PS-AsODN and gel retardation analysis showed that the vectorized formulations with SP and TP were relatively stable in FBS concentrations of 1 and 25% after 4 h incubation.

Antisense targeting for therapeutic and imaging purposes using AsODNs requires hybridization to the target mRNA following internalization and thus a cytosolic rather than nuclear delivery pathway for the AsODN is required. It is known that naked ODNs remain sequestered in endosomal and lysosomal vesicles after endocytosis, where they may undergo degradation by nucleases or cell elimination by exocytosis³⁴ and only a very limited amount might be able to access the target in the cytosol. Vectorization, therefore, not only aims to increase cellular uptake, but also to modify the intracellular distribution after internalization. Synthetic polymers, such as cationic polymers and cationic lipids, are being increasingly investigated for their potential for site specific delivery of ODN to tumor cells³⁵.

Vectorization of ODN with cationic lipids and polymers leads to the formation of complexes with high affinity to cell membranes. Cell entry takes place by non-specific interaction with the membrane enhancing adsorptive endocytosis. Endosomal escape of the ODN is accomplished by destabilization of endosomes or lysosomes at acidic pH. For Lipofectin complexes destabilization is accomplished by the “helper lipid” DOPE and after escape DOTMA directs the ODN preferentially to the nucleus³¹. However, if the AsODN remains complexed with the cationic lipids, hybridization to target mRNA in the cytosol will be compromised. When cationic block copolymers, such as SP and TP, are used for ODN delivery endosomal escape takes place by the so-called “proton sponge effect”, the buffering

capacity of the polymers causing increased osmotic pressure, and therefore swelling and rupture or leakage of the vesicle with release of their cargo into the cytosol³⁶. Especially, for the novel vectors used in this study an efficient endosomal escape and a specific cytosolic delivery pattern has been shown¹⁴. Due to the higher propensity for dissociation more “free” AsODN might be available to hybridize to the target mRNA enhancing the potential for antisense targeting.

In the cellular uptake studies besides slightly increased uptake of naked [¹⁸F]PS-AsODN in HCT116 versus 80S4 cells at later timepoint after incubation, we observed a higher cell uptake of [¹⁸F]PS-AsODN in HCT116 versus 80S4 cells when delivered with SP and TP. This finding could potentially reflect the better endosomal escape and cytosolic delivery of the AsODN by these vectors compared to others, but has to be confirmed by further experiments, comparing the uptake of naked and vectorized radiolabeled AsODN and RdODN in the two cell lines.

While cationic lipids such as Lipofectin are very effective for transfection *in vitro*, they have limiting disadvantages *in vivo* related to cytotoxicity, inhibition of the delivery in the presence of serum and size limiting cellular membrane permeability³¹. In contrast biodegradable block copolymers may prove to be better candidates for delivery of AsODN for molecular targeting purposes due to their lower toxicity, relatively small particle size <100 nm and especially more efficient ODN delivery into the cytosol¹⁴. The availability of a radiolabeled probe additionally provides the possibility to monitor and quantify the fate of the AsODN *in vivo*.

Future investigations in the field of ODN delivery will have to elucidate the influence of the delivery agent on the pharmacokinetics and biodistribution, and especially the intracellular trafficking of ODN³⁷. One major challenge will be to overcome the accumulation of ODN in endosomes and inefficient release to the cytosol. In this perspective the described novel vectors or an improved generation hold high promise to overcome some of the obstacles currently encountered with ODN delivery.

Conclusions

Initial studies *in vitro* with radiofluorinated PS-AsODN demonstrated improved cellular uptake by using novel synthetic vectors for delivery when compared to naked PS-AsODN. Our results support further investigations, which are ongoing, comparing the uptake of radiolabeled AsODN and RdODN in the two cell lines, as well as studying the subcellular distribution and efflux of naked and vectorized PS-AsODN labeled with fluorescent probes. Even though a lower vector assisted PS-AsODN uptake was observed *in vitro* with SP and TP in comparison with Lipofectin, these novel block copolymers still promise advantages in terms of safe administration *in vivo*, serum integrity and improved subcellular distribution with high potential for application for antisense targeting approaches.

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Figure 1.
HPLC profile of $[^{18}\text{F}]$ PS-AsODN after NAP purification: A) UV-trace of the unreacted PS-AsODN precursor at 254 nm (dots); B) radiochromatogram of $[^{18}\text{F}]$ PS-AsODN (solid line).

SP **TP**

Figure 2.
Binding capacity of non-radiolabeled PS-AsODN by gel electrophoresis with 14 increasing vector to ODN weight ratios: 0:1 (1), 0.5:1 (2), 1:1 (3), 2:1 (4), 4:1 (5), 8:1 (6), 16:1 (7), 32:1 (8).

Figure 3. Stability based on gel electrophoresis of naked non-radiolabeled PS-AsODN and the different vectorized formulations after 4 h incubation in PBS containing 1% and 25% FBS.

Figure 4. Immunofluorescence analysis showing enhanced p21 expression in HCT116 cells (A: DAPI spectrum, B: FITC spectrum) and deficient p21 expression in 80S4 cells (C: DAPI spectrum, D: FITC spectrum) after gamma irradiation.

Figure 5. Cell uptake of naked [¹⁸F]PS-AsODN on HCT116 and 80S4 cells and blocking of uptake with non-radiolabeled PS-RdODN and PS-AsODN (mean±SD, *n*=3).

Figure 6.

Cell uptake of naked and vectorized [¹⁸F]PS-AsODN by HCT116 cells and 80S4 cells at 2 h and 4 h after incubation (mean±SD, *n*=3; *significantly increased versus 80S4 cells, *p*<0.05).

Scheme 1.

Biodegradable polyamine grafted PEO-*b*-PCL based copolymers, grafted with spermine (SP) and tetraethylenepentamine (TP) used for vectorization.

Table 1

Relative cell associated radioactivity of naked and vectorized [^{18}F]PS-AsODN after incubation at 4°C or co-incubation with heparin sulfate. Data is expressed as percentage of uptake in relation to the cell associated activity at 37°C without co-incubation with heparin sulfate (mean $\pm$ SD, $n=3$). Absolute values used for reference are presented in Fig. 6.

naked	Lipofectin	SP	TP	
HCT116		Relative cell associated radioactivity (%)		
4°C	52.9 ± 13.1	90.7 ± 1.2	77.8 ± 3.5	82.4 ± 7.5
Heparin sulfate	128.2 ± 13.3	44.4 ± 3.6	22.6 ± 1.8	21.7 ± 1.0
80SA		Relative cell associated radioactivity (%)		
4°C	53.5 ± 2.9	80.3 ± 9.7	77.3 ± 4.3	79.1 ± 0.7
Heparin sulfate	123.6 ± 18.7	37.9 ± 1.3	23.5 ± 2.6	24.9 ± 4.1

Table 2

Relative retained cell uptake of naked and vectorized [^{18}F]PS-AsODN following cell membrane treatment with 0.04 M sodium acetate pH 4.5 or PBS/10% FBS. Data is expressed as percentage of radioactivity relative to the cell associated radioactivity without membrane treatment (mean $\pm$ SD, $n=3$). Absolute values used for reference are presented in Fig. 6.

naked	Lipofectin	SP	TP	
HCT116		Relative retained cell uptake (%)		
Sodium acetate	66.2 ± 6.6	82.8 ± 6.1	74.4 ± 2.7	64.4 ± 4.1
PBS/10%FBS	43.4 ± 1.4	89.4 ± 0.9	38.6 ± 0.9	47.4 ± 2.2
80SA		Relative retained cell uptake (%)		
Sodium acetate	82.9 ± 1.6	92.8 ± 0.6	84.9 ± 0.4	84.8 ± 1.9
PBS/10%FBS	39.7 ± 1.8	86.9 ± 0.6	31.6 ± 0.5	38.6 ± 2.8